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Technical Note

Fresh versus frozen PMBC using the SARS CoV-2 T-SPOT.*COVID* test. Which works best?

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ABSTRACT

With the onset of the SARS-CoV-2 pandemic and subsequent vaccination programme, a need has arisen to check for the development of T lymphocyte immunity against the virus. The SARS CoV-2 T-SPOT.*COVID* test measures the level of T cell immunity and has been used extensively in our laboratory over the last 6 months. Whilst this kit has been designed to be used on freshly isolated human peripheral blood mononuclear cells (PBMC), the use of frozen cells would improve clinical utility. To this end we have directly compared the use of fresh and frozen PBMC in this assay. Using healthy control blood along with renal and liver transplant patient samples we have shown that results with frozen cells are generally comparable to those from fresh cells in many, but not all samples tested, and that it is important to assess PBMC cell number and viability in thawed samples before proceeding in order to be able to interpret these results correctly.

1. Introduction

A key requirement arising from the SARS-CoV-2 pandemic is the need to assess if individuals have obtained immunity, following either infection or vaccination, necessary to give some level of protection against future re-infections. This is especially important given mutations identified in the recently described Omicron variant (Kandeel et al., 2021). Whilst general screening of the public is not feasible, certain patient groups may benefit from some measurement of post vaccine immunological monitoring. In particular patients who may be immunocompromised, either due to a primary immunodeficiency or following treatment for an underlying condition (patients on immunosuppressive drug treatments) (Steve Woodle et al., 2021; Brill et al., 2021; Stumpf et al., n.d.) may benefit from this test. With this in mind, we have recently assessed the use of the T-SPOT.*COVID* test (Oxford Immunotec) for use in our lab to assess the T cell responses obtained from secondary immunodeficiency patient groups post vaccination and a cohort of healthy control volunteers. We additionally included a patient with persistent COVID as a positive test subject who was expected to respond well to both COVID antigen groups.

Our initial testing used freshly isolated human PBMC and the results obtained were broadly in line with expectations given the infection/vaccination status of the people tested, and has proved a useful tool especially when paired with SARS-CoV-2 antibody assays. The T cell assay was designed to be used on freshly isolated human PBMC preparations; however, there are situations where the use of frozen PBMC would be beneficial. Given that it is unclear whether the results obtained from frozen samples reflect the true T cell status of the individuals tested, we have set out to compare results from the T-SPOT.*COVID* kit using paired fresh and frozen PBMC from healthy individuals. As the use of this assay is likely to be most informative in patient groups, where a level of immunosuppression potentially exits, we included a small cohort of renal and liver transplant patients in addition to healthy control samples. Results from this small study indicate that with some adjustments to interpretation, it is generally safe to use frozen samples although care needs to be taken in interpretation of non-reactive data.

2. Methods

The cohort tested in this study included; 24 samples from 18 healthy

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volunteers, 7 post liver transplant patients, 6 post renal transplant patients, 1 stem cell transplant patient, 1 rheumatoid arthritis patient and 1 patient with "persistent Covid" (prolonged PCR confirmed COVID infection). Where vaccination status was recorded, transplant patients had received two doses of either Astra Zeneca or Pfizer vaccines and were tested post dose 2. Volunteers were tested at various stages post dose 1 (V1), dose 2 (V2) or booster dose (V3) and one healthy control was tested pre vaccine and post vaccine (Tables 1 and 2).

In all cases, 10 mL of peripheral blood in lithium heparin was obtained from each individual along with an EDTA sample from the same individuals. PBMC were isolated from the lithium heparin samples using Lympholyte H (Cedar Lane) following manufacturer's instructions and the final cell pellet resuspended in AIM-V serum free media (Oxford Immunotec). Cell viability and density was assessed using 0.4% trypan blue (Sigma), a haemocytometer and microscopy. PBMC were made up to 2.5×10^6/mL in AIM-V serum free media and 100 μL of cells were added to wells of the T-SPOT.*COVID* filter plate (Oxford Immuntec), the antigens were added and the assay completed as per the manufacturer's instruction (T-SPOT, n.d.). Where excess PBMC were isolated, they were pelleted by centrifugation and resuspended at approximately 5×10^6/ml in sterile heat inactivated FBS (Invitrogen) with 10% DMSO (Sigma). Resuspended cells were frozen in 1 mL aliquots at -80 °C using a *Mr Frosty* for initial freezing. Prior to inclusion into the T-SPOT.*COVID* assay, frozen vials were thawed quickly using a 37 °C water bath. Cells were subsequently washed twice in TC199 tissue culture media (Gibco) by centrifugation (1100 rpm for 10 min), resuspended in AIM-V serum free tissue culture media and the cell viability and density assessed. Assay preparation then followed the same protocol as outlined for the freshly isolated PBMC. All unused frozen samples were destroyed at the end of the study.

At the end of the ELISPOT assay, spots were counted in the ELISPOT wells using a DX-1 digital microscope (Veho) and the MicoCapture Plus software (V2.0, Veho). The number of spots from the AIM-V media negative control wells were subtracted from the number of spots in the antigen wells. Using this method and following manufacturer's recommendations and criteria for spot counting, four or less spots was considered non-reactive, 5–7 was borderline and 8 or greater was considered reactive.

Table 1
Fresh vs Frozen Healthy Controls. R – Reactive, NR – Non-reactive, BL – Borderline.

Designation	Time post vaccine	Spot No: Fresh	Spot No: Frozen	Interpretation Fresh/Frozen	Agreement
HC 1.1	10.4 wks post V2	18	13	R/R	Y
HC 1.2	2.3 wks post V3	>40	>40	R/R	Y
HC 2.1	**10.6 wks post V2**	**7**	**4**	**BL/NR**	**N**
HC 2.2	**26.7 wks post V2**	**10**	**1**	**R/NR**	**N**
HC2.3	2.7 wks post V3	8	12	R/R	Y
HC 3	8 wks post V1	28	>40	R/R	Y
HC4	7.6 wks post V1	7	5	BL/BL	Y
HC 5.1	7.7 wks post V1	1	2	NR/NR	Y
HC 5.2	2.7 wks post V2	19	10	R/R	Y
HC 6.1	Unvaccinated	0	1	NR/NR	Y
HC 6.2	18.4 wks post V2	1	4	NR/NR	Y
HC 7	Post V3 (vaccine date unknown)	>40	>40	R/R	Y
HC8	**2.7 wks post v3**	**14**	**6**	**R/BL**	**N**
HC 9	Post V3 (vaccine date unknown)	36	>40	R/R	Y
HC 10.1	3.7 wks post V3	15	20	R/R	Y
HC 10.2	**6.6 wks post V3**	**5**	**2**	**BL/NR**	**N**
HC 11	**32.1 wks post V2**	**4**	**5**	**NR/BL**	**N**
HC 12	7.6 wks post V3	5	7	BL/BL	Y
HC 13	30.6 wks post V2	7	5	BL/BL	Y
HC 14	4.9 wks post V3	7	7	BL/BL	Y
HC 15	11.7 wks post V3	19	28	R/R	Y
HC 16	4 wks post V3	>40	>40	R/R	Y
HC 17	**11.7 wks post V3**	**13**	**7**	**R/BL**	**N**
HC 18	**12 wks post V3**	**10**	**3**	**R/NR**	**N**

3. Results

We initially considered the samples from healthy individuals ($n = 24$) to ascertain if we could detect any difference in spot count between samples tested fresh and those from cells that had been frozen and thawed, but otherwise taken at the same time (Table 1). Blood samples were taken from 18 individuals with five volunteers having samples taken on more than one occasion (healthy controls 1, 5, 6 and 10 had 2 separate samples taken and HC 2 had samples taken on three separate occasions). Of the 24 samples tested fresh and after freezing 17/24 gave the same final interpretive result using the Oxford Immunotec counting guidance (Fig. 1 shows examples of representative images from 4 individuals), 7/24 samples, however, gave different results. Out of these, 2/7 moved from a borderline result to non-reactive following freezing (HC 2.1 and 10.2), 1/7 was non-reactive prior to freezing and borderline post freezing (HC 11), 2/7 were reactive prior to freezing and non-reactive following freezing (HC 2.2 and 18) and 2/7 were initially reactive but became borderline following freezing (HC 8 and 17). Lymphocyte counts and cell viability from each of these samples was adequate for testing post freezing.

We additionally assessed the effect of freezing patient samples from two small cohorts of post renal ($n = 6$) and post hepatic ($n = 7$) transplant patients to observe the effects of freezing PBMC from these patients prior to use in the T-SPOT.*COVID* test (Table 2). Of the liver transplant patients tested only 2/7 samples were comparable between fresh and frozen cells. However, closer analysis of this data also indicated that the loss of COVID spike response following freezing of 3/7 samples was also reflected in the low PHA responses (Liver Tx 1, 2 and 4) thus invalidating the antigen specific responses from these samples. Furthermore, two additional patients (Liver Tx 1 and 4) had a low T-lymphocyte count pre-freezing which would have impacted the observed response to the Covid spike antigens.

Freezing PBMCs appeared to have less of an impact on post renal transplant samples, with 5/6 samples giving similar results pre and post freezing. The one post frozen sample which didn't respond also had a poor PHA response (along with a low post-freeze cell count) indicating that the results from this sample were not safe and a repeat sample would be required.

Interestingly four individuals gave a reactive response to the nucleocapsid antigen preparation in addition to Spike peptides indicating prior exposure to SARS-CoV-2 virus. Two of these individuals were healthy volunteers who had prior PCR confirmed infection (HC 9 and 16), one was a kidney transplant patient (Kid Tx 1) and one was the individual with persistent Covid. Of these four, three had similar results for the response to nucleocapsid following freezing. The liver transplant sample, however, was reactive when tested on fresh PBMCs but non-reactive following freezing, although this was likely a reflection of the lower cell count in the frozen sample (as indicated by the poorer PHA response).

4. Discussion

In this study we have assessed the use of frozen PBMC when using the

Table 2
Fresh vs Frozen on samples from immunocompromised participants. R – Reactive, NR – Non-reactive, BL – Borderline.

Designation	Time post vaccine	Spot No: Fresh	Spot No: Frozen	Interpretation Fresh/Frozen	Agree	Comments
Liver Tx 1	23.4 wks post V2	**8**	**3**	**R/NR**	**N**	**Low T Lymphocyte PHA spot count low**
Liver Tx 2	**13.9 wks post V2**	**9**	**3**	**R/NR**	**N**	**PHA spot count low**
Liver Tx 3	10.1 wks post V2	9	10	R/R	Y	
Liver Tx 4	**18.6 wks post V2**	**12**	**1**	**R/NR**	**N**	**Low T Lymphocyte PHA spot count low**
Liver Tx 5	21.6 wks post V2	>40	32	R/R	Y	
Liver Tx 6	**25.4 wks post V2**	**14**	**0**	**R/NR**	**N**	
Liver Tx 7	**21.4 wks post V2**	**7**	**0**	**BL/NR**	**N**	
Kidney Tx 1	16.1 wks post V2	>40	13	R/R	Y	PHA spot count low
Kidney Tx 2	18.1 wks post V2	14	13	R/R	Y	
Kidney Tx 3	**16 wks post V2**	**10**	**3**	**R/NR**	**N**	**PHA spot count low Below recommended cell count post freezing**
Kidney Tx 4	Post V2 (vaccine date unknown)	9	9	R/R	Y	
Kidney Tx 5	10 wks post V2	9	11	R/R	Y	
Kidney Tx 6	15 wks post V2	16	8	R/R	Y	
RA volunteer	**4.4 wks post V3**	**6**	**8**	**BL/R**	**N**	
Post BMT	**3.7 wks post V3**	**6**	**0**	**BL/NR**	**N**	
Persistent Covid	Pre vaccine, post infection	>40	>40	R/R	Y	

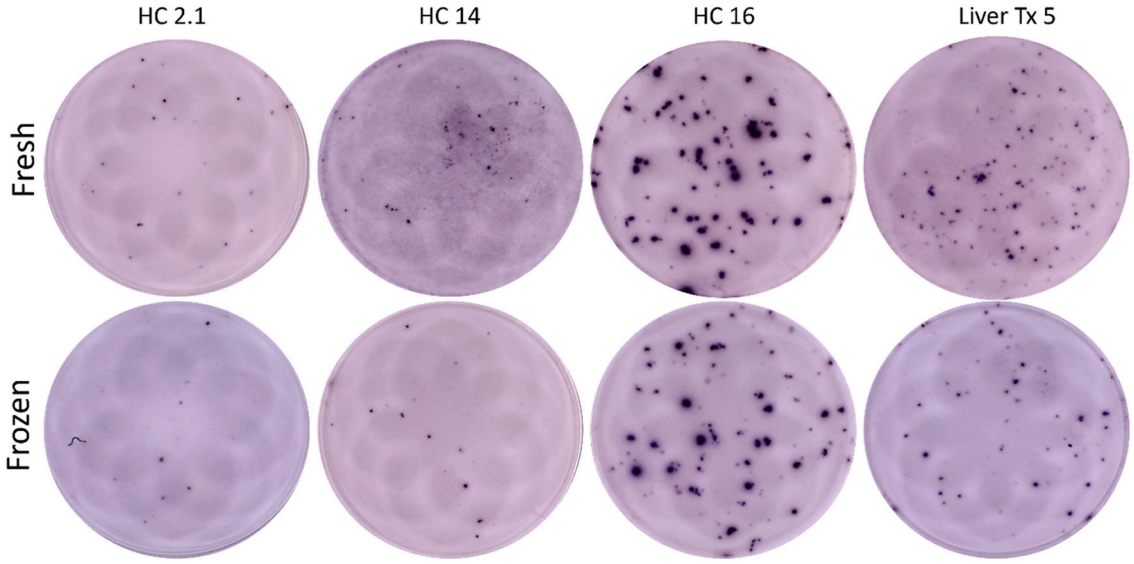

Fig. 1. SARS-CoV-2 Spike specific T cells using fresh vs frozen PBMC in 4 individuals.
PBMC from 4 individuals were isolated (used either fresh or after freezing as indicated) and stimulated for 16 h with SAR-Cov-2 spike peptides prior to testing for interferon gamma production using the T-SPOT.COVID test . Spot number was counted and results from fresh and frozen compared.

Oxford Immunotec T-SPOT.*COVID* test by comparing results obtained from paired fresh and frozen PBMCs. According to manufacturer's instructions, freshly isolated PBMC should be used; here we aimed to determine whether results obtained from frozen samples were safe to report. Although ideally the manufacturer recommended method is preferable, it is not always possible to arrange receipt of freshly taken samples to a laboratory from clinical areas. Additionally, study samples collected off site may have to be frozen to aid transport to the laboratory. In these situations, the use of frozen PBMCs would be beneficial and would also allow samples to be batched together, allowing a greater degree of testing flexibility and improving sample throughput through the laboratory. It is important, however, that the results obtained when previously frozen PBMC are used are robust, and similar to those obtained using a fresh sample. The samples used in this study include healthy volunteers as well as a group of patients with secondary immunodeficiencies; these were used to reflect the patient groups for whom the SARS CoV-2 T-SPOT.*COVID* assay would be clinically relevant.

Although, in many cases, there was a consensus between fresh and frozen healthy control cells in terms of spot count interpretation, we did note in a number of samples where the frozen samples gave a significantly lower result when compared to fresh cells. Whilst this was initially alarming, we were partially reassured to observe that in most (although not all) cases these differences could be explained by methodological reasons. However, the inclusion of some important checks is important to ensure that only safe results are reported. A key parameter within this assay is the cell density, which should be 2.5×10^6/mL. Whilst a slight reduction from this density may not adversely affect the results, any significant deviance may lead to a poor response, especially to the antigen preparations. In the event of a reduced PHA response being noted, with the absence of an antigen response, the results should not be reported, and a repeat sample should be requested. Further to this, the use of a viable cell dye (in this case trypan blue) should be used to ensure that the final cell density is accurate and that cells thawed from previous freezing are still viable. It is also useful to run the SARS-CoV-2 antibody screen alongside the T cell assay. Again, where there is

discrepancy between the antibody levels and the T cell response (especially where frozen cells are used), then a repeat T cell test, preferably using fresh cells should be considered.

Where there appears to be a discrepancy in the interpretation of the results in our cohort (borderline in fresh to non-reactive in frozen etc), we observed that the spot counts were generally around the borderline numbers and in some cases the results only differed by a few spots. We would therefore suggest that where frozen PBMCs results in borderline or non-reactive responses, a repeat sample is requested and tested fresh where possible. With this in mind, we would also recommend increasing the range which triggers a request for repeat samples to counts between 4 and 10 rather than the manufacturer recommended 5–7.

Ideally it is also useful to assess lymphocyte subset numbers (lymphocyte markers especially $CD4^+$ and $CD8^+$ T cells) test prior to testing the Covid antigen response to ensure that sufficient T cells are present to allow a reactive result. This is especially important in patient groups such as transplant patients (both solid organ and post haematopoietic stem cell transplantation) where lymphocyte depletion therapy (for examples alemtuzimab/basiliximab in renal transplant recipients) are used post-transplant leading to temporary but initially severe lymphopenia.

In conclusion this study has shown that, provided care is taken (especially in respect to cell density and viability) frozen PBMC can be used in the Oxford Immunotec SARS CoV-2 T-SPOT.*COVID* test. A slightly amended spot number for equivocal counts should be used (between 4 and 10) and where there is any doubt a repeat sample (preferably processed fresh) should be requested.

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